Phaeohyphomycosis caused by Cochliobolus hawaiiensis in a Camel Farm in Saudi Arabia: An Emerging Disease

Abstract

Phaeohyphomycosis describes subcutaneous lesions caused by dematiaceous fungi, brown-pigmented mould. In a camel farm in Saudi Arabia, the owner complained of cases of skin infection among camels. Lesions persisted after sarcoptic mange outbreak which was treated until the infection resolved. General examination revealed that four camels were affected showing alopecia, erythema, numerous small subcutaneous nodules and brownish blackish crusts. To collect specimens, affected areas were disinfected with 70% ethyl alcohol and deep were taken. Skin scrapings were prepared in 10% potassium hydroxide for microbiological examination. Cultures were done onto Sabouraud dextrose agar (SDA) with chloramphenicol 0.5 mg per ml, incubated at 30^0 C and another set with chloramphenicol 0.5 mg per ml and cycloheximide (Sigma), 0.4 mg/L, incubated at 27^0 C and 37^0 C. Molecular mycology analysis was done by Polymerase Chain Reaction (PCR) on internal transcribed spacer (ITS) gene using ITS1 and ITS4 primers.

Microscopic examination indicated it was negative for dermatophytes. It showed brownish to black, septate hyphae arranged as arthro-hyphae, and black yeast-like particles. Cultures yielded multiple, velvety, gray colonies turning brownish black later. Lactophenol cotton-blue smears revealed septate, branched hyphae that are dark brown in colour1.5–5 μ wide. Conidiophores are septate, unbranched with flexuose apexes, bearing brown, multi-septate, cylindrical conidia. ITS gene sequence analysis confirmed the isolate from camel skin scrapings to be *Cochliobolus hawaiiensis*. The case is interesting as this represents, to the best of our knowledge, the first authenticated report of *C. hawaiiensis* in animals from a tropical country.

Keywords: Phaeohyphomycosis; dromedary camel; skin infection; *Cochliobolus hawaiiensis*; Saudi Arabia.

Introduction

Phaeohyphomycosis is a general term used to describe solitary subcutaneous lesions caused by brown-pigmented mould known as dematiaceous fungi. If left untreated these lesions slowly increase in size to form a painless abscess. Clinical infections caused by dematiaceous fungi are classified as chromomycoses, mycetomas, or phaeohyphomycoses. The dematiaceous fungi infections are most common in warm, humid, tropical and subtropical climates. Phaeohyphomycoses, first described by Ajello et al. in 1974 [1], are emerging and opportunistic diseases that may infect man and animals. Dematiaceous fungi are characterized by the presence of pale to dark brown melanin-like pigment in their cell walls and live in rotting vegetables, decaying wood, dust and soil [2, 3].

Cochliobolus Drechsler (1934) [4] species, with asexual states in Bipolaris Shoemaker (1959) [5] and Curvularia Boedijin (1933) [6], are important plant pathogens affecting many species [7, 8]. A few species of them are causative agents of vertebrate opportunistic infections [9].

The genus *Curvularia* (family: Pleosporaceae, order: Pleosporales) known as hyphomycete fungus was established by Boedijn and typified by *C. lunata* (Wakker) Boedijn belongs in Pleosporaceae, Pleosporales [10]. Recently, many Curvularia species with a great diversity of species including relevant phytopathogenic, animal and human pathogenic fungi, have been reported. Multi-locus sequence analysis was more feasible than microscopic identification of species. Hence, morphological characterization and illustration of species from different countries with three novel species were presented [11].

Bipolaris hawaiiensis is a darkly pigmented fungus that can cause phaeohyphomycosis in animals and man (Ajello et al., 1974) [1]. It is widely distributed in nature and most frequently related to plant material, grasses and soil.

Different Bipolaris species that can infect humans include *Bipolaris australiensis*, *Bipolaris hawaiiensis.* Bipolaris species are rarely implicated in cutaneous infections. Robb et al. recently reported superficial cutaneous Bipolaris in 3 patients without predisposing medical conditions. Straka et al. [12] reported a case of a non-healing cutaneous ulcer due to Bipolaris species in a pancytopenic patient presenting with acute leukemia following traumatic injury. Inoculation of minor wounds with fungi from contaminated sources appears to be closely associated with wound exacerbation and infection. Superficial cutaneous infection by Alternaria spp. or Bipolaris spp. was reported [13]. A case of cutaneous phaeohyphomycosis in an Antillean manatee *Trichechus manatus manatus* caused by *B. hawaiiensis* was described. Blackish skin lesions were observed in an Antillean manatee calf held captive in Brazil. Direct examination of skin scraping from the affected areas revealed the presence of dematiaceous hyphae. Culture of skin fragments led to the isolation and subsequent identification of *B. hawaiiensis* as the etiologic agent. Infections by Bipolaris spp. are rare in animals, and this is the first report of *B. hawaiiensis* in veterinary medicine [14]. A 63-year-old man with a history of trauma and saw dust in the left eye was diagnosed as fungal keratitis caused by *B. hawaiiensis* [15]. *B. australiensis, B. hawaiiensis* and *B. spicifera* were documented to cause infection at different sites of human body at Riyadh Military Hospital, Kingdom of Saudi Arabia (KSA) by conventional methods only. Eight cases from nasal site infection, four cases from wound and burn sites, three cases of post-operation and two case each from lung and skin infections were recorded during the study period between 2004 and 2008. This is the first report of Bipolaris human infection from KSA [16], however, the infection was not described from animals in the KSA.

The camel (*Camelus dromedaries*) play significant roles in social and economic development for people in different geographical sites. It has been considered an aid to man for thousands of years by providing meat, milk, leather, fibre, fuel, transportation (packing, riding). Camels are also used for draft purposes, pulling ploughs and wagons. Infectious diseases, poor nutrition and traditional management systems have restricted their full utilization. As camels are usually reared under harsh environment, suitable for propagation and transmission, parasitic, bacterial and

mycotic skin diseases are fairly common [17]. Here, cases of phaeohyphomycosis in a camel farm in Al-Ahsa, Eastern Region, KSA, is presented.

Materials and Methods

In a camel farm, the owner complained that there were cases of skin infection among some adult and young camels. General physical examination was conducted on each camel in the herd which revealed that four camels were affected.

Physical examination revealed that the animals had alopecia, erythema, numerous small subcutaneous nodules and brownish blackish crusts with hyperkeratosis (Fig. 1) on neck, abdominal region and the thighs. The owner stated that the camels have been affected with sarcoptic mange which was treated until the lesions disappeared.

Fig. 1 Skin lesions of a camel infected with phaeohyphomycosis showing roughened hairy skin with alopecia, scales, numerous small subcutaneous nodules and brownish blackish crusts

To collect specimens, affected areas were disinfected with 70% ethyl alcohol and with sterile scalpels deep skin and hair scrapings were taken from the lesions' margin in sterile Petri-dishes.

Microbiological Examination:

Microbiological analysis was done by preparation of wet mounts in 10% potassium hydroxide for examination with light microscope. Cultures were done onto Sabouraud dextrose agar (SDA) (Oxoid) slopes and Petri-dishes with chloramphenicol 0.5 mg per ml, incubated at 30^0 C and another set with chloramphenicol 0.5 mg per ml and cycloheximide (Sigma), 0.4 mg/L, incubated at 27^0 C and 37^0 C. Subcultures were done onto potato dextrose agar (PDA) plates.

Molecular Mycology Analysis:

ITS (internal transcribed spacer) gene sequence analysis:
DNA extraction

Genomic DNA was extracted by scraping about 0.5g of mycelia from the surface of a PDA plate. The mycelium was grounded for 3–5 min in a sterile pestle and mortar. Then 600 µl extraction buffer (2 %(w/v) CTAB, 100 mM Tris–HCl, 1.4 M NaCl, 20

mM EDTA, pH 8.0) was added and transferred to 1.5 ml micro centrifuge tubes. The solution was incubated at 60 °C with a gentle swirling. The mixture was centrifuged at 12,000 rpm for 15 min at 25 °C followed by chloroform extraction repeatedly. DNA was precipitated with isopropanol and centrifuged at 12,000 rpm for 15 min at 25 °C. The precipitate was treated with 70 % ethanol centrifuged at 12,000 rpm for 5 min at 25 °C. DNA was dried under a regular air flow for 20 min and stored in −20 °C.

PCR on ITS (internal transcribed spacer) gene using ITS1 and ITS4 primers [18] was performed by Macrogen Inc. (Seoul, South Korea).

Results:

Direct Examination: Tricho-gram was done on KOH wet mounts to exclude dermatophytosis which was negative. Microscopic examination showed brownish to black round, irregular, septate hyphae arranged as arthro-hyphae within epithelial cells (Fig. 2), and black yeast-like particles.

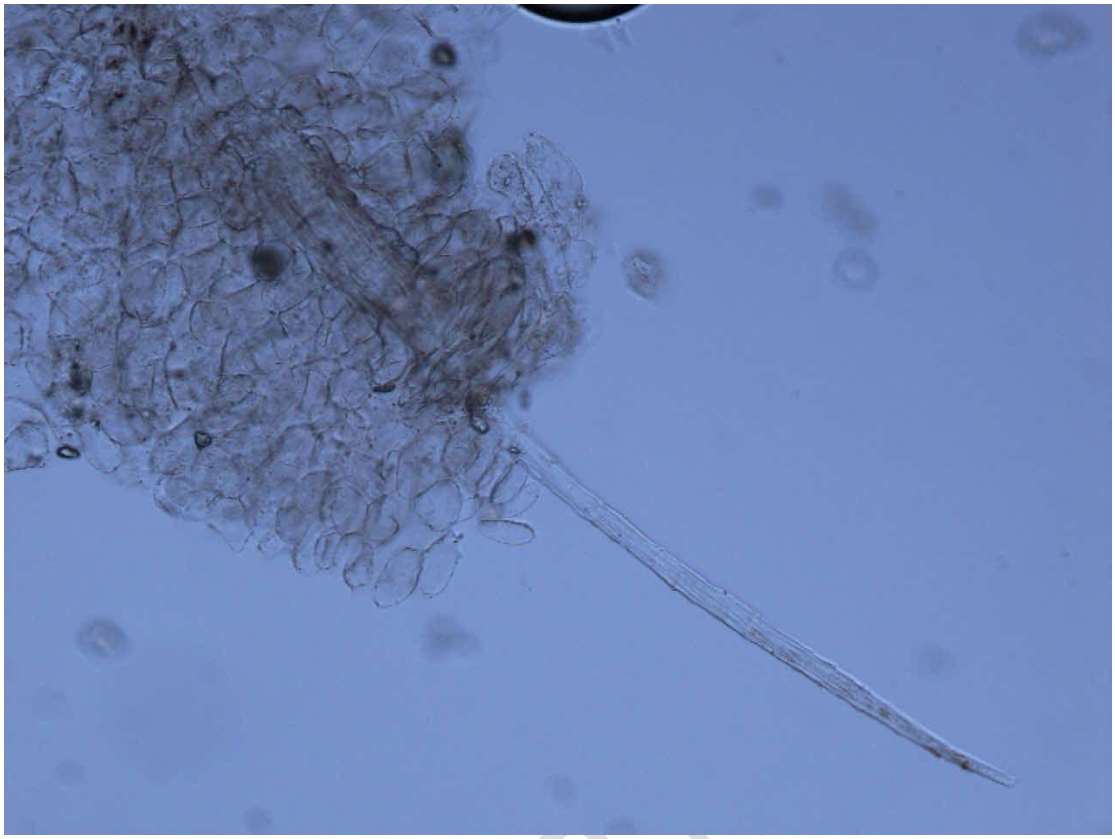

Fig. 2 Skin scrapings of camel infected with phaeohyphomycosis shows brownish to black round, irregular, septate hyphae arranged as arthro-hyphae within epithelial cells, and black yeast-like

Cultures on Sabouraud dextrose agar (SDA) + chloramphenicol and SDA + chloramphenicol + cycloheximide gave colonies of a dematiaceous fungus but growth on the later medium was not luxuriant. After three days of incubation at 28°C and at 37°C, SDA plate cultures, inoculated with camel skin scrapings, yielded multiple, velvety, gray colonies turning brownish black later (Fig. 3). From primary culture

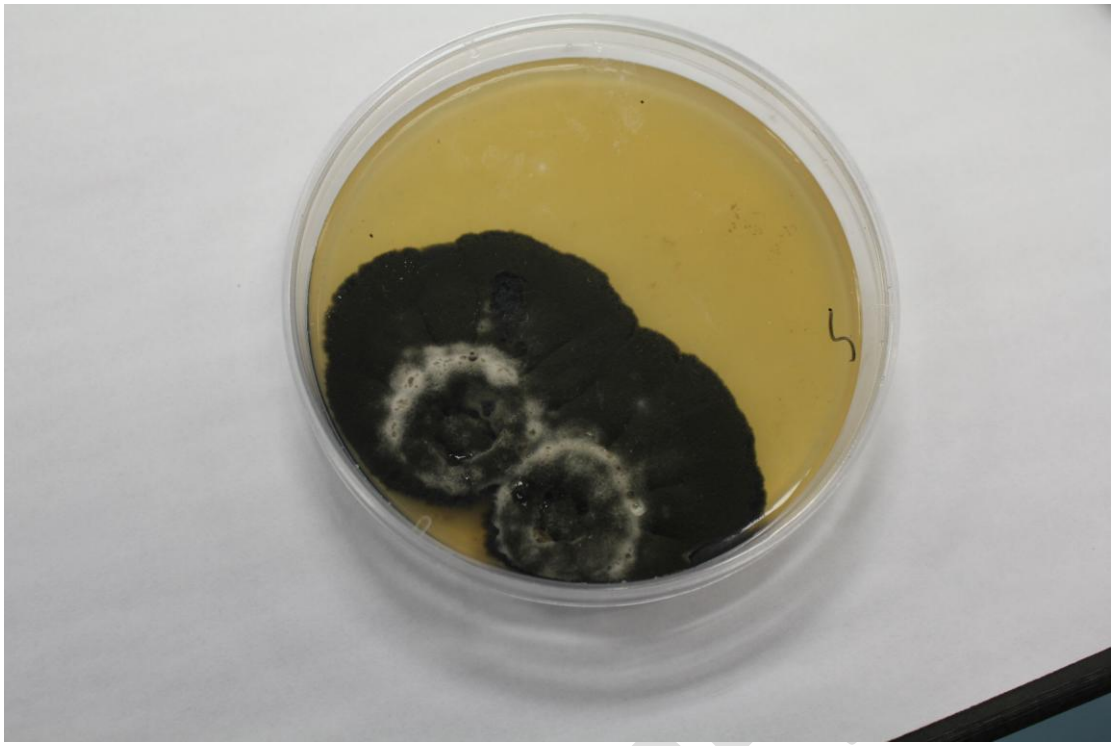

Fig. 3 Dematiaceous fungus colonies on Sabouraud's dextrose agar + chloramphenicol, inoculated with camel skin scrapings, after three days of incubation at 28°C and at 37°C, yielded multiple, velvety, gray colonies turning brownish black later

sub- cultures of the isolate on potato dextrose agar (PDA) were done. Wet mounts were stained with lactophenol cotton-blue and microscopically examined. They revealed septate, branched vegetative hyphae that are dark brown in colour1.5–5 μm wide. Conidiophores arise singly or in small groups which are septate, unbranched with flexuose apexes, bearing thick-walled, brown, multiseptate conidia. Conidia are produced from the apex of an unbranched conidiophore, straight cylindrical broad in the middle and tapering towards the rounded ends, with 3-4 distoseptate and the hilum is not clear. (Fig. 4).

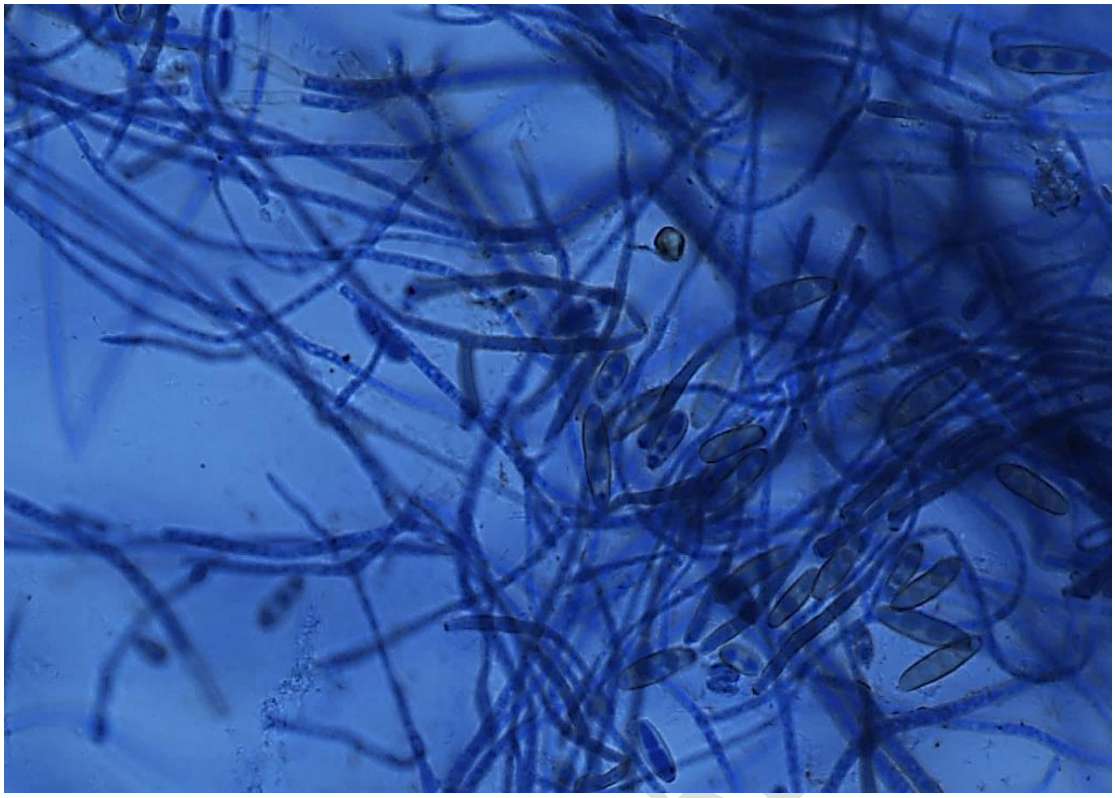

Fig. 4 Wet mounts of Cochliobolus hawaiiensis stained with lactophenol cotton-blue, there is septate, branched vegetative hyphae, unbranched conidiophores bearing straight cylindrical conidia with 3-4 distoseptate

ITS Gene Sequence

Cochliobolus hawaiiensis genomic DNA containing 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene (partial), strain VPCI-98-09
Length=575

Score = 1020 bits (552), Expect = 0.0
Identities = 572/580 (99%), Gaps = 8/580 (1%)
Strand=Plus/Plus

Score = 444 bits (240), Expect = 9e-121
Identities = 291/313 (93%), Gaps = 14/313 (4%)
Strand=Plus/Plus

Discussion

Clinical forms of phaeohyphomycoses range from superficial infection to subcutaneous and systemic involvement. The present case of phaeohyphomycosis was caused by C. hawaiiensis, the teleomorph of Curvularia spp. and Bipolaris spp. Identification of the causative agent to the species level was completed using both phenotypic and molecular techniques. The case is interesting as this represents, to the best of our knowledge, the first authenticated report of Cochliobolus hawaiiensis in animals from a tropical country.

Mycological study of wet mounts of the culture, showed the presence of fertile perithecia of C. hawaiiensis. The ascomatal neck morphologies are generally regarded as a generic feature of Cochliobolus [19, 20]. Studies with *C. hawaiiensis* showed that this is not a fixed feature as considerable variation may be encountered in the neck of this species. Ascomata neck can vary from long cylindrical to short or in some strains is absent even when grown in the same conditions [19].

Molecular techniques for species confirmation is quite helpful for the fungi with ambiguous microscopic picture. *Bipolaris spicifera* differs from *B. australiensis* by its 3-distoseptate conidia which show a pale area at the base, just above the scar, while those of *B. australiensis* are usually 3-4(-5)-distoseptate, and the pale basal area is absent. The ITS sequences of the type strains of both species showed a similarity of 97.8%. *Bipolaris hawaiiensis* is morphologically distinguished from *B. spicifera* and *B. australiensis* by its 5(-7)-distoseptate narrow conidia (up to 7 µm wide), while those of *B. australiensis* and *B. spicifera* are 6-11µm and 9-14µm wide, respectively.

A study in the KSA, recorded 23 clinical isolates of Bipolaris, obtained from different anatomical sites [16]. The most frequent isolates, identified by conventional methods only, were *B. spicifera*, *B. australiensis* and *B. hawaiiensis*. This finding correlates generally with previous reports in the literature [21, 22]. Finding of the present study confirms the above-mentioned human study.

We hypothesized that cumulative effects of various predisposing factors may lead to emergence of hitherto unknown infections in an animal species. Epidemiologically, it could be attributed to pseudoepidemics due to infection of a number of animals from the same source [23]. Curvularia spp. have a widespread distribution on plant materials, in soil and air. together with a wide diversity of fungi. Hence, to qualify as opportunistic pathogens for camel skin, dematiaceous fungi need to inhibit fungal competitors *in situ*. An important predisposing factor gives substance to the argument; camels in the farm have been recovered from severe sarcoptic mange infection. Malnutrition may affect calcium, iron, magnesium and phosphorus concentrations. Deficiency was associated with decreased immune status and also have clinical relevance with poor outcome in diseased and poor conditioned camels.

Control measures may include application of environmental hygienic measures in camel farms, correct nutritional deficiency and prompt treatment of skin diseases.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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